

Coordinated activation of VEGFR-1 and VEGFR-2 is a potent arteriogenic stimulus leading to enhancement of regional perfusion

Alexander Babiak^a, Anke-Mira Schumm^a, Christoph Wangler^a, Marios Loukas^a, Jianbo Wu^a,
Saskia Dombrowski^a, Christiane Matuschek^b, Jörg Kotzerke^{b,1},
Christoph Dehio^c, Johannes Waltenberger^{a,d,*}

^aDepartment of Internal Medicine II, Ulm University Medical Center, Ulm Germany

^bDepartment of Nuclear Medicine, Ulm University Medical Center, Ulm, Germany

^cBiozentrum of the University of Basel, Basel, Switzerland

^dDepartment of Interventional Cardiology, University Hospital Maastricht, and Cardiovascular Research Institute Maastricht (CARIM), Maastricht, The Netherlands

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Abstract

Objective: The process of arteriogenesis is driven by various growth factors including vascular endothelial growth factor (VEGF)-A, which mediates its activity through VEGFR-2 (Flk-1/KDR) on endothelial cells and through VEGFR-1 (Flt-1) on endothelial cells and monocytes. The purpose of this study was to identify which of the VEGF receptors are involved in arteriogenesis *in vivo*. **Methods:** Collateral vessel growth was induced by femoral artery ligation in a mouse model of hindlimb ischemia. Following ligation, Balb/c mice were treated with different growth factors (VEGF-A, VEGF-E, PlGF-2, VEGF-E plus PlGF-2 or VEGF-A plus PlGF-2, activating either VEGFR-1, VEGFR-2, or both). After 1 week of treatment, hindlimb perfusion was assessed by perfusion scintigraphy using Tc-99m-MIBI. **Results:** The strongest improvement of regional perfusion was achieved by simultaneous activation of VEGFR-1 and VEGFR-2, using either VEGF-A or VEGF-A plus PlGF-2, with elevation of relative perfusion in the ischemic limbs from 0.61 to 0.83. The partial restoration in perfusion was associated with morphological changes typical for arteriogenesis. Moreover, specific inhibition of both VEGF-receptors using ZK 202650 resulted in a significant inhibition of arteriogenesis, indicating an active role of the VEGF system in compensatory arteriogenesis. **Conclusion:** The coordinated activation of both VEGFR-1 and VEGFR-2 represents a more potent arteriogenic stimulus compared to the isolated activation of either one of these two receptors. These data imply that the activation of both monocytes and endothelial cells is necessary to obtain a maximal VEGF-induced activation of arteriogenesis.

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1. Introduction

The growth of preformed collateral arteries is regarded as the most important mechanism to enhance regional perfusion

Abbreviations: VEGFR-1, vascular endothelial growth factor receptor-1, Flt-1; VEGFR-2, vascular endothelial growth factor receptor-2, Klf-1/KDR; PlGF-2, placenta-derived growth factor-2; Tc-99m-MIBI, hexakis(2-methoxyisobutylisonitrile)-technetium-99m

* Corresponding author. Department of Interventional Cardiology, University Hospital Maastricht, P. Debyelaan 25, 6202 AZ Maastricht, The Netherlands. Tel.: +31-43-3875106; fax: +31-43-3875104.

E-mail address: j.waltenberger@cardio.azm.nl (C. Dehio).

¹ New address: Klinik und Poliklinik für Nuklearmedizin, Fetscherstr. 74, 01307 Dresden, Germany.

following the obstruction or ligation of the feeding artery. This process is called arteriogenesis and describes the active growth of arterioles into arteries allowing a significant increase of regional blood flow [1]. While the cellular and molecular basis of arteriogenesis has partly been clarified [1,2], the relative functional importance of certain growth factors including vascular endothelial growth factor (VEGF) remains to be elucidated. Likewise, the involvement of monocytes has been demonstrated in the process of arteriogenesis and there are models explaining the functional impact of monocytes during arteriogenesis as reservoirs and bioreactors of various growth factors [3]. However, it remains to be shown to which degree monocytes are contributing to the growth of arterioles.

VEGF-A has successfully been used to stimulate vascular growth in a large variety of different models. In a rabbit model of hindlimb ischemia, therapeutic application of VEGF-A was shown to stimulate limb perfusion by increasing capillary density (angiogenesis) and collateral size [4]. However, the mechanism of action of VEGF-A in the stimulation of arteriogenesis remains to be elucidated.

VEGF-A can bind to both VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). Based on the distribution pattern of these two VEGF receptors, VEGF-A stimulates not only endothelial cells that express both of these receptors [5], but also monocytes that carry functional VEGFR-1 capable of mediating monocyte chemotaxis [6]. The recent discovery of the *orf*-virus encoded VEGF-E that can induce angiogenesis by exclusive binding to VEGFR-2 [7–9] opens the possibility of the isolated activation of VEGFR-2 in endothelial cells. Whether this is sufficient to enhance limb perfusion by stimulating angiogenesis and/or arteriogenesis remains to be shown. In addition, placenta growth factor-2 (PlGF-2), another member of the VEGF family, can be used to stimulate VEGFR-1 on both cell types [10], which was sufficient to stimulate arteriogenesis in PlGF^{-/-} mice [11]. Finally, a direct effect of VEGF-A on activated smooth muscle cells cannot be excluded as activated smooth muscle cells in injured arteries had been shown to express VEGFR-1 [12].

In the present study, we investigated whether VEGF-E (and therefore activation of VEGFR-2) is sufficient to induce arteriogenesis. Moreover, we studied, whether the action of VEGF-A can further be enhanced by additional stimulation of VEGFR-1 using PlGF-2. To proof the role of VEGF and its receptors, the tyrosine kinases of both receptors were inhibited using either ZK 202650 or specific neutralizing antibodies MF 1 and DC 101. To answer these questions, a mouse model was established, which precisely allows the quantitation of blood flow in hind limb scintigraphy.

2. Materials and methods

2.1. Mouse hindlimb model

A mouse model of hindlimb ischemia was established. All animal work was approved by the governmental authority (Regierungspräsidium Tübingen) and supervised by institutional animal protection officials. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). We used 12 weeks old Balb/C mice weighting between 26 and 30 g. The mice were kept pathogen-free in groups of five in a strict 12-h night and day cycle. In brief, unilateral ligation of the right femoral artery was performed under short-term anaesthesia. The anaesthetic contained 104 mg/kg Ketamin (Ketanest®

S, Parke-Davis) and 16 mg/kg Xylazin (Rompun® 2%, Bayer) and was given as a single bolus of 0.27 ml into the peritoneal cavity.

Prior to perfusion analysis, exactly 7 days following ligation (see below), the animals were treated for the whole period with repeated injections of either recombinant growth factor or carrier solution (0.1 mg/ml mouse serum albumin (Sigma) in phosphate buffered saline) into the tail vein every 12 h starting immediately after femoral artery ligation. The mice were given either VEGF-A (500 ng bid), VEGF-E (500 ng bid), PlGF-2 (1000 ng bid), a combination of VEGF-E and PlGF-2 or a combination of VEGF-A and PlGF-2 (same doses as in the case of isolated application). VEGF-A and PlGF-2 were purchased from Reliatech, Germany. Recombinant VEGF-E was generated in *Escherichia coli* as previously described [7]. The biological activity of all the different recombinant proteins has been verified by in vitro experiments prior to application to animals. Each group consisted of a total of seven animals.

Another series of mice was treated with ZK 202650 (Schering, Berlin). The substance was applied subcutaneously, starting 2 days prior to ligation and repeated every other day. Three groups were investigated, 20 mg/kg ZK202650 ($n=4$), 50 mg/kg ($n=7$) and placebo ($n=7$). In another series, neutralizing antibodies directed against VEGFR-1 (MF-1, $n=9$) and VEGFR-2 (DC-101, $n=10$), both from Imclone, were applied into the peritoneal cavity to block the activity of VEGF.

2.2. In vivo perfusion measurement using scintigraphy

Perfusion analysis was previously described in rats and validated using coloured microspheres [13]. In the present study, perfusion analysis was transferred for the first time on a mouse model. The method was validated by perfusion assessment in a group ($n=7$) of non-operated and non-treated animals, which had equal perfusion in both hind limbs (ratio 0.98 ± 0.11). Furthermore, a series with sham operated animals vs. animals with a ligated limb (0.54 ± 0.23 vs. 0.91 ± 0.11 , $p < 0.04$) was scanned. Perfusion analysis was performed 7 days after femoral artery ligation. The discriminatory power of this method was maximal at day 7 and better than on day 4 or on day 14 (own, unpublished data). Quantitative perfusion imaging was performed using i.v. injection of Hexakis(2-methoxyisobutylisonitrile)-technetium-99m (Tc-99m-MIBI) (Cardiolite®, Dupont Pharma), a lipophilic perfusion marker (bolus of 200 μ l containing a dose of 50–60 MBq) and quantitation on a gamma camera (Basicam®, Siemens, using the ICON software package). In order to exclude interindividual differences as well as investigator-dependent bias, the ratios between ligated and non-ligated limb were measured in a standardised fashion (see definition of target area in Fig. 1A). Three investigators confirmed placement of the regions of interest prior to calculation. Statistics were done using ANOVA. The data are presented as the ratio of

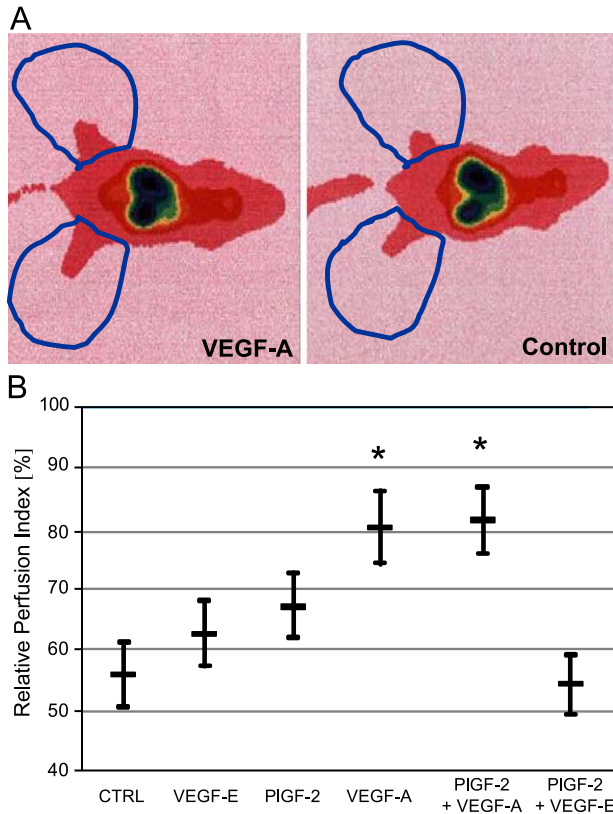


Fig. 1. Quantitative assessment of mouse hind limb perfusion 7 days following ligation of the right femoral artery. (A) Representative perfusion images (with the target area marked blue) are shown for a VEGF-A treated mouse (left) and for an untreated mouse 7 days after ligation of the femoral artery (right). (B) The relative perfusion index is given as an average of seven animals in each group showing least square mean and 95% CI. * $p < 0.05$ compared to control using ANOVA.

perfusion in the ligated over perfusion in the non-ligated limb including the 95 % confidence interval.

2.3. Macro- and micromorphological evaluation of collateral arteries

2.3.1. Estimation of a collateral score

Sixteen hours following perfusion imaging, the mice were sacrificed during anaesthesia. The aorta was cannulated through the left ventricle using a 24 G catheter (Insyte® 24 G \times 0.75, Becton Dickinson) followed by injection of 2 ml saline, 1 ml heparin (5000 I.U. Heparin-Natrium, B. Braun Melsungen), 2 ml adenosine (6 mg) for vasodilation and 1 ml of a bismuth contrast agent to visualise the vascular system. The contrast agent was a mixture of bismuth (III) nitrate and gelatine, produced in our laboratory. Photographs were taken using a Contax 167 attached to the operating microscope (OPMI-1, Carl Zeiss, Germany) (compare Fig. 3A–D). The extent of the collateral artery growth was semiquantitatively estimated by five investigators using a standard assessment protocol (see Fig. 3E, F).

2.3.2. Immunofluorescence analysis of monocytes during arteriogenesis

Subsequently, the collateral arteries were dissected out and immediately deep-frozen using liquid nitrogen. Histological analysis was performed for the presence of CD11b (Mac-1 α chain) positive leukocytes using the FITC-conjugated (Flourescein Isothiocyanate Isomer I conjugated) anti-mouse CD11b antibody (PharMingen International) and standard protocols. Photomicrographs were taken using a fluorescence microscope (Leica), the collateral diameter was quantitatively assessed.

3. Results

3.1. The activation of both VEGFR-1 and VEGFR-2 using VEGF-A is necessary to induce significant enhancement of limb perfusion following femoral artery ligation

Recombinant VEGF and PlGF were applied in our hind limb model at concentrations that had already been reported to result in a positive biological response elsewhere. The strongest improvement of regional perfusion was achieved by simultaneous activation of VEGFR-1 and VEGFR-2 using VEGF-A, applied for 7 days twice a day. Relative perfusion 1 week after femoral artery ligation could be elevated from 0.61 (95% CI: 0.52–0.70) in the control group to 0.83 (95% CI: 0.74–0.92) in the VEGF-A treated group ($p = 0.0088$) and to 0.82 (95% CI: 0.73–0.91) in the group receiving both VEGF-A and PlGF-2 ($p = 0.0022$) (Fig. 1B). The isolated activation of VEGFR-1 using PlGF-2 resulted in a moderately elevated relative perfusion of 0.66 (95% CI:

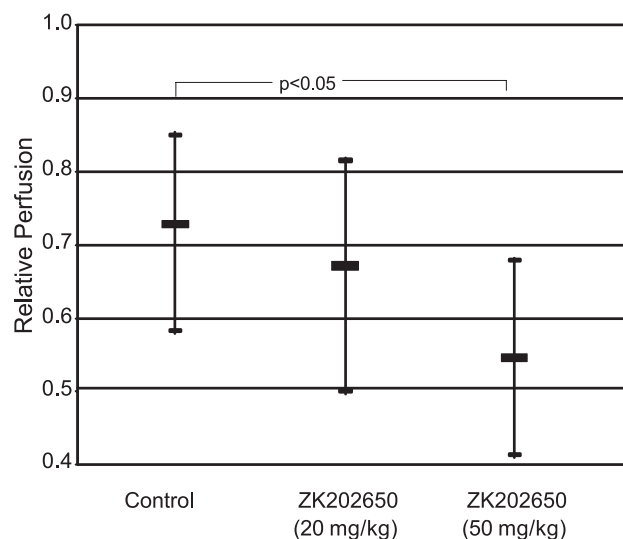


Fig. 2. Relative perfusion of right over left hind limb after seven days of ligation of the right femoral artery was assessed by scintigraphy. Three groups receiving placebo, ZK 202650 20 mg/kg and ZK 202650 50 mg/kg were compared. The mean perfusion and S.D. is presented. At 50 mg/kg, the restoration of perfusion was significantly decreased compared to placebo.

0.57–0.75) ($p>0.4$), while the isolated activation of VEGFR-2 using VEGF-E resulted in a relative perfusion of 0.65 (95% CI: 0.56–0.73) ($p>0.5$). In both instances of activation of a single VEGF receptor, perfusion in the ischemic limb was not significantly different from the untreated control group. The same was observed for the simultaneous injection of PlGF-2 and VEGF-E, which did not result in the enhancement of limb perfusion. Primary data obtained on the gamma camera already indicate reduced perfusion of the ligated hindlimb in the control group without growth factor stimulation (Fig. 1A, right panel).

3.2. The activation of VEGF receptors is involved in compensatory arteriogenesis

The application of ZK 202650, an inhibitor of VEGF receptor tyrosine kinase activity, resulted in a reduction of

perfusion on day 7 following ligation. While restoration of blood flow was not compromised in the control group (0.72 ± 0.14), application of ZK 202650 significantly decreased perfusion in a concentration-dependent fashion reading 0.5 ± 0.14 ($p=0.0055$) at a concentration of 50 mg/kg. (0.55 ± 0.14 , $p=0.0055$) (Fig. 2). Likewise, experiments were performed using neutralizing antibodies to both VEGF-receptors. No effect on perfusion was apparent in this series of experiments (data not shown).

3.3. Enhanced limb perfusion 7 days following femoral artery ligation is associated with arteriogenesis

The growth of collateral arteries, i.e. extent of arteriogenesis, can be visualised by microphotography and semi-quantitatively analysed following injection of bismuth-containing contrast agent into the aorta. Typical examples

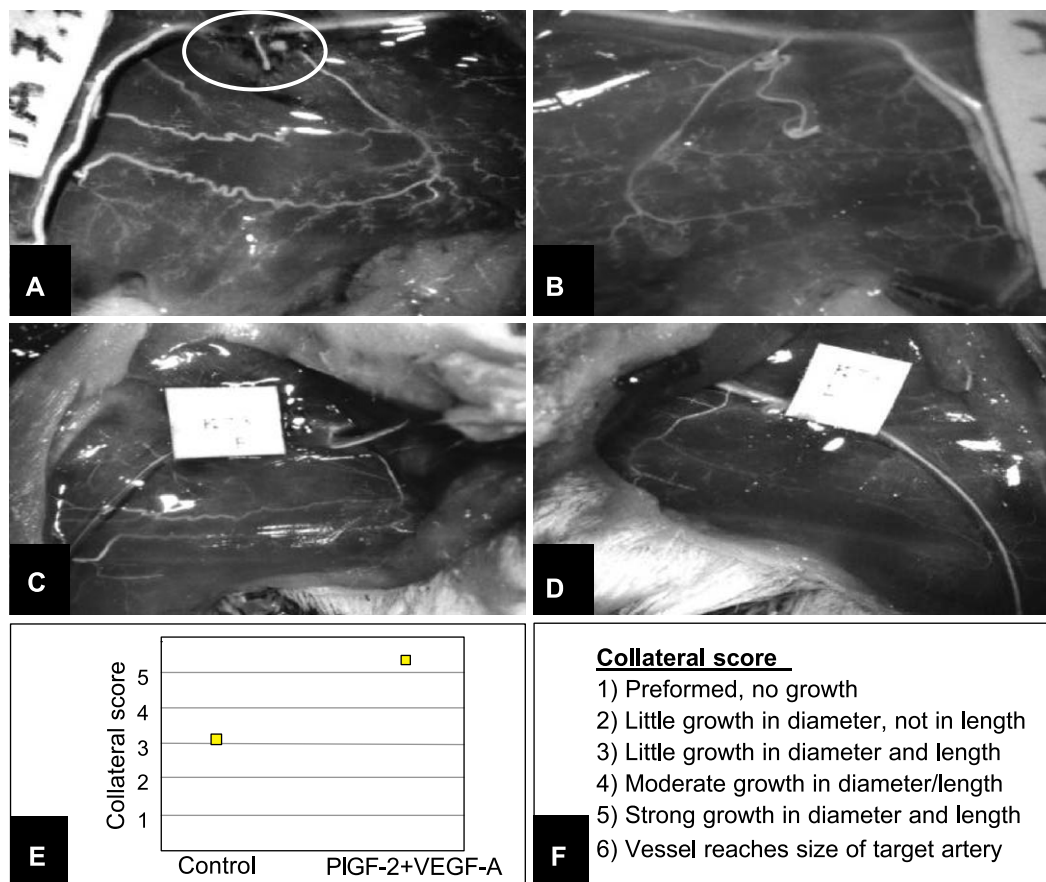


Fig. 3. Anatomy of collateral vessels in the mouse hindlimb model. Visualisation by microphotography after injection of bismuth contrast agent into the aorta 7 days following ligation of the right femoral artery (A, C). The situation of the corresponding contralateral, non-ligated control side is shown for comparison (B, D). (A) Ligation of the femoral artery and repeated iv-injections of VEGF-A is accompanied from impressive growth of collateral vessels. The diameter of the collateral vessel reaches the one of the distal femoral artery. The typical pattern of tortuosity in the grown collateral vessel is noted. The site of the ligation is highlighted by a circle. (B) No collateral artery growth can be seen on the non-ligated contralateral side of the mouse receiving VEGF-A. (C) Similar to the setting in A, however, the animal received repeated injections of saline buffer. Please notice that there is some growth of the collateral vessel as compared to the non-ligated contralateral control (D). However, the diameter of the collateral vessel is much smaller than in the VEGF-A-treated animals (A). (E,F) Semiquantitative assessment of the size of collateral arteries in mice secondary to femoral artery ligation and iv-treatment with growth factors is given on a scale ranging from 1 (preformed, not-grown collateral vessel) to 6 (collateral artery measuring the same size as the feeding artery). Representative data are shown for the PlGF-2+VEGF-A group, which had shown the strongest effect of the enhancement of perfusion in the ligated limb.

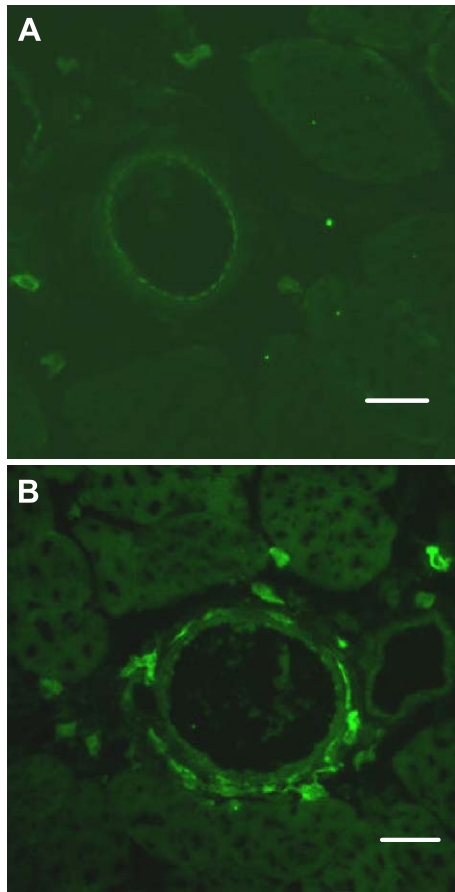


Fig. 4. Mac-1 positive leukocytes in a preformed collateral artery (A) and infiltrating the growing collateral artery 7 days following ligation of the femoral artery (B). Bars indicate 20 μ m. Original magnification: 100 \times .

are shown in Fig. 3A–D. The average collateral score measured 3 in the control group and 5.2 in the PIGF-2 plus VEGF-A group, which was the group with the strongest improvement following growth factor application (Fig. 3E), which measured the highest level of limb perfusion. Mac-1 positive leukocytes are hardly detectable in a preformed collateral artery (Fig. 4A), while there are abundant CD11b (Mac-1 α chain) positive cells infiltrating the wall of the growing collateral artery 7 days following ligation of the femoral artery (Fig. 4B). This cellular infiltration is associated with an enhancement of collateral diameter from 0.079 ± 0.012 to 0.13 ± 0.013 mm ($n=20$, $p<0.05$), as assessed by histomorphometry.

4. Discussion

Using a mouse hindlimb model of regional ischemia, we have found that the concerted activation of both VEGFR-1 and VEGFR-2 using prolonged and repeated administration of VEGF-A was sufficient to enhance limb perfusion and to stimulate arteriogenesis. In contrast, the isolated activation of VEGFR-1 using PIGF-2 or the isolated activation of

VEGFR-2 using VEGF-E was not sufficient to significantly enhance perfusion of the ischemic extremity in Balb/C mice. The positive effect of VEGF-A was on a similar level as the effect of the combined application of VEGF-A and PIGF-2. These data suggest that VEGF-A is a potent arteriogenic stimulus and that the coordinated activation of VEGFR-1 and VEGFR-2 is necessary for the stimulation of arteriogenesis in the hindlimb model.

The simultaneous, but isolated, i.e. not concerted activation of VEGFR-1 using PIGF-2 and of VEGFR-2 using VEGF-E, was not associated with a detectable arteriogenic stimulus in our model. There are at least two possible explanations for this finding: (1) In contrast to the potent arteriogenic stimulus VEGF-A, the combination of VEGF-E plus PIGF-2 is unable to form VEGF receptor heterodimers. Such heterodimers had long been postulated [5] and have recently been demonstrated [14]. Our *in vivo* data are compatible with the interesting hypothesis that a relevant arteriogenic stimulus requires the formation of VEGFR-1/VEGFR-2 heterodimers. (2) We cannot exclude the possibility that the combination of PIGF-2 and VEGF-E was lacking a functional effect in our model on the basis of inadequate dosing or inadequate action of VEGF-E secondary to rapid degradation. However, no reagents are currently available to prove this issue.

Assuming a transitor function of VEGF-receptors during arteriogenesis. The isolated activation of VEGFR-1 using PIGF-2 is not sufficient in our Balb/C wild-type mice to enhance arteriogenesis and tissue perfusion. This situation is different from the situation in PIGF^{-/-} mice, however, which show an impaired arteriogenic response secondary to acute ischemia that can be overcome by the application of PIGF-2 [11]. This could be explained by a functional interaction between VEGFR-1 and VEGFR-2 inside the endothelial cell. The reduced level of VEGFR-1 activation in PIGF^{-/-} mice is associated with a reduced functional activity of VEGFR-2, which is not the case in wild-type mice, as we have just shown [14].

The most potent arteriogenic stimulus in our model were VEGF-A and the combination of VEGF-A and PIGF-2, respectively. It activates both VEGFR-1 and VEGFR-2. Though VEGFR-1 is being expressed on endothelial cells as well as on monocytes, both cell systems seem to play a crucial role in regulating arteriogenesis.

Monocytes have been suggested to play a critical role in the process of arteriogenesis, and we can provide further evidence in our study. This is sustained by the observation that both receptors (VEGFR-1 and VEGFR-2) need to be activated in order to obtain a significant effect on the improvement of limb perfusion through growing collateral arteries. However, the data of our study clearly suggest that the activation of monocytes via VEGFR-1 is not sufficient to induce arteriogenesis as the application of PIGF-2 (or the application of PIGF-2 plus VEGF-E) did not result in enhanced limb perfusion. In conse-

quence, the activation of the endothelium should play a crucial role in the induction of arteriogenesis. In contrast to monocytes, endothelial activation is mediated via both VEGFR-1 and VEGFR-2 [5]. VEGFR-1 has been recognized as an important positive mediator of the VEGF-signal in endothelial cells [14,15] (and own, unpublished data). Moreover, the role of VEGFR-1 on activated vascular smooth muscle cells in arteriogenesis deserves further analysis.

One argument in favour of a critical role of monocytes in the process of arteriogenesis came from the observation that monocyte activation using LPS or MCP-1 stimulates arteriogenesis [3]. It remains to be shown, however, whether endogenous MCP-1 plays a role in arteriogenesis. This question is critical in the light of the recent observations that (i) MCP-1 directly stimulates endothelial cells [16,17] and that (ii) MCP-1 can initiate a potent angiogenic stimulus [18]. It will be an important task of future studies to definitely clarify the role of monocytes in specific and the role of circulating cells in general in the process of arteriogenesis.

The selective blockage of the VEGF receptor tyrosine kinases using ZK 202650 resulted in a decreased perfusion after 7 days indicating a decrease of the arteriogenic response. These data clearly suggest that VEGF plays an active role in compensatory arteriogenesis in vivo. In contrast, the application of monoclonal antibodies neutralizing the activity of either VEGFR-1 or VEGFR-2 or both showed no effect on perfusion. It is likely that these antibodies were inefficient due to either inappropriate pharmacokinetics, degradation or poor local tissue availability.

The quantitative analysis of limb perfusion has until recently not been possible in mice. In larger animals such as rabbits, perfusion analysis was possible when radioactive or coloured microspheres were injected into the arterial system or when flow measurement devices were used. We have now adopted Tc-99m-MIBI analysis for quantitative analysis of limb perfusion in the mouse, a method that can easily and reproducibly be applied in the mouse model. Moreover, longitudinal studies are possible using this non-invasive method. Scintigraphy reflects perfusion of the hind limb over the full thickness of the limb. This greatly differs from laser Doppler scanning [19], which assesses tissue perfusion only to a depth of approximately 200 µm. It is rather unlikely to assume that skin perfusion really reflects limb perfusion. Therefore, a nuclear scan using TC-99m-MIBI should be one of the most valid non-invasive methods to assess hindlimb perfusion. It is important to note that the scintigraphic enhancement of perfusion was paralleled by morphological changes reflecting true arteriogenesis.

Our study demonstrates that the mouse hindlimb model is suitable to document the functional consequences of therapeutic arteriogenesis that occur within the first week after femoral artery ligation. The stimulation of arteriogenesis using VEGF-A or VEGF-A plus PlGF-2 was safe and no side effects such as hypotension or edema forma-

tion could be observed in our model. Taken together the isolated activation of either one of the VEGF receptors did not result in a significant enhancement of perfusion in the mouse hindlimb model. In contrast, the simultaneous activation of both VEGFR-1 and VEGFR-2, possibly by heterodimer formation or by coordinated activation of both homodimers, is associated with a significant enhancement of regional perfusion. Finally, there is good evidence that the VEGF system is an important mediator of endogenous compensatory arteriogenesis, i.e. in the absence of therapeutic stimulation.

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